**Self-assembled arrays**

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**Stepwise Self-Assembly of a Protein Nanoarray from a Nanoimprinted Poly(Ethyleneglycol) Hydrogel**

Bong Kuk Lee,* Hea Yeon Lee,* Pilnam Kim, Kahp Y. Suh, Jeong Hyun Seo, Hyung Joon Cha, and Tomoji Kawai*

Protein micro- and nanoarrays have emerged as high-throughput screening tools for a variety of diagnostic assays, such as tissue engineering, pharmacology, and proteomics.[1-10] Miniaturized biochips are currently being developed for the integration of electrical, optical, and physical measurements with fluid handling.[11-14] It has been shown that use of small quantities of sample can substantially improve efficiency, speed, and the accuracy of miniaturized detection technologies in a fast, high-resolution, low-cost manner.[15-17]

In fabricating protein micro- and nanoarrays as components of biochips, some key elements, such as protein address density and activity, need to be considered for practical applications. Various techniques have been lithographies and related scanning probe patterning reported using top-down principles, including dip-pen methods,[21-23] ink-jet printing,[24-25] soft lithography,[26-29] and nanoimprint lithography.[30,31] Focused ion beam, electron beam, and dip-pen lithographies are capable of forming patterns at nanoscale resolution, but are relatively delicate serial processes and thus lack scalability. In contrast, soft and nanoimprint lithographies offer the advantages of high throughput, low cost, and high reproducibility. Furthermore, these methods are capable of creating nanopatterns with sub-100-nm features over large areas.

In this Communication, nanoimprint lithography (NIL) was used to fabricate protein nanoarrays with an inert poly(ethylene glycol) (PEG) polymer as the resist material. Previous studies have demonstrated that NIL can form protein arrays with a minimum feature below 100 nm.[32,33] A polymer lift-off process should be used because of the non-specific binding of protein molecules to the resist surface; however, complete lift-off of the residual resist is problematic.[32] Consequently, this limitation complicates the imprinting process and restricts the use of this method in biological laboratories. It is believed that use of an inert material to prevent nonspecific binding would greatly simplify the imprinting process, leaving behind selective linker or probe arrays within micro- or nanowells.

The most widely used inert materials are uncharged PEG-based polymers,[28,31] and self-assembled monolayers (SAMs).[34,35,36] The latter is incompatible with the NIL process owing to difficulties in direct imprinting. Among the various PEG-based polymers, ultraviolet (UV)-curable PEG, such as PEG diacrylate (PEGDA);[33,35] PEG dimethacrylate,[35] a block copolymer of polycaprolactone and PEG,[34] and acrylate-terminated star PEG,[36] have been recently used for fabricating micro- or nanopatterns on transparent substrates using UV-embossing. Because these polymers on the master mold can be cured by UV irradiation from the top of transparent substrates,[33-36] this method is very similar to UV-nanoimprint lithography (UV-NIL)[37] and suitable for the patterning of transparent substrates with a UV-curable polymer. UV-NIL enables the patterning of both transparent and nontransparent substrates, in which the polymer can be cured by UV irradiation from the top of transparent mold.[37] However, in the construction of protein nanoarrays, no reports have described the fabrication of nanopatterns on nontransparent substrates with a PEG-based polymer using UV-NIL.

In this study, we developed a very effective and widely applicable method for fabricating nanopatterns of a PEG hydrogel for protein nanoarrays. The nanopatterns of non-biodegradable PEGDA hydrogel were fabricated on a gold substrate by UV-NIL (Scheme 1a). The protein nanoarray with a nanoimprinted PEGDA hydrogel was constructed by stepwise self-assembly from biotinylated PEG SAM, streptavidin (SA), and biotinylated antihuman serum albumin (anti-bHSA) (Scheme 1b).

Scheme 1a shows the UV-NIL process of fabricating nanopatterns of PEGDA hydrogel on a gold substrate. The superior mechanical and chemical stability of the patterns are key features for the success of future applications. It has been reported that a PEGDA pattern, that has not been modified with an adhesion-promoting layer, could be easily delaminated from the substrate upon hydration owing to swelling of the cross-linked PEGDA matrices.[38] Simple treatment of the surface with an acrylated SAM effectively prevented delamination.[38,39] Therefore, the gold substrate was initially modified with N,N'-bis(acryloyl) cystamine

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(BAC) SAM to enhance adhesion between PEGDA and the substrate (Scheme 1a, stage I). The terminal acrylate of BAC SAM on the gold substrate was co-crosslinked, with the PEGDA containing 1 wt% 2,2'-dimethoxy-2-phenylacetophenone as a photoinitiator,[35,39] by UV irradiation (Scheme 1a, stage II). To fabricate robust patterns, non-biodegradable PEGDAs with low molecular weights (PEGDA258: MW = 258; PEGDA575: MW = 575) were used.[35,38–40]

The streptavidin (SA)–biotin reaction system was used to construct protein nanoarrays into the nanowell (NW) of the PEGDA575 hydrogel (Scheme 1b). Owing to the high affinity and specificity of the SA–biotin interaction (dissociation constant $K_d = 4 \times 10^{-14}$ M),[41] this reaction system is a helpful tool in many immunoassays and therefore commonly used for protein patterning.[29–31,35,42] The gold substrates in the PEGDA NWs were initially modified with thiol-functionalized PEG mixtures composed of methoxy PEG thiol (mPEG-S) and biotinylated PEG disulfide (bPEG-S) (Scheme 1b, stage I). mPEG-S and bPEG-S act as polymer cushions to prevent direct protein–substrate contact, resulting in denaturation, and as a biospecific ligand for SA immobilization, respectively. Because they do not adsorb on the surface of PEGDA hydrogel, it is assumed that these thiol-functionalized PEGs are selectively grafted onto the gold substrates. The selective protein nanoarray in the PEGDA NW, modified with the mPEG-S/bPEG-S mixed SAM, was formed by stepwise self-assembly with SA as a linker protein (Scheme 1b, stage II) and anti-bHSA as a model probe protein to detect the target protein (Scheme 1b, stage III).

Figure 1 shows height and cross-sectional tapping-mode atomic force microscopy (TM-AFM) images of the 500-nm patterns of PEGDA575 hydrogel on gold substrates. Regarding the nanopattern of PEGDA575 prebaked at 80°C for 10 min under cleanroom conditions, some dimples (20 nm in height) were observed on the surface of the PEGDA575 hydrogel, despite the nanopattern being created by UV-NIL (Figure 1a and d). The dimples disappeared with an increase in the prebaking temperature to 100°C for 10 min (Figure 1b and e). This indicates that solvent retention is one of the reasons for the surface depression of PEGDA575 hydrogel. The imprinted depth (99.1 nm) of the PEGDA575 NW was almost the same as the mold height (100 nm), suggesting that the mold patterns were faithfully transferred by UV-NIL. The imprinted residual layer of PEGDA575 was about 10 nm, as determined by an Alpha-Step 500 surface profiler (Tencor Instruments Inc., Mountain View, CA, USA) after oxygen (O2) reactive ion etching (RIE) with the mask. The residual layers of PEGDA575 and BAC SAM in the NWs were successfully removed by O2 RIE (Figure 1c and f). The NW depth of the PEGDA575 hydrogel was controlled by changing the O2 RIE time and was about 57 nm. Compared to the PEGDA575 hydrogel, the PEGDA258 hydrogel has higher mechanical strength;[35,40] however, we found that fabricating the nanopatterns of PEGDA258 hydrogel using UV-NIL was very difficult. Dimples were also present on the surface of the PEGDA258 hydrogel, but micropatterns larger than 3 μm were successfully replicated without dimples (Supporting Information). Unfortunately, for patterns less than 1 μm, these dimples were not removed by changing the prebaking temperature, prepolymer concentration, or UV irradiation dose. Therefore, the PEGDA575 nanopatterns were used for protein nanoarrays throughout the experiments.

For the stepwise protein nanoarray, dry PEGDA575 patterns of 57.3 nm NW depths were used. A phosphate-buffered saline (PBS) solution was selected for AFM observations in the aqueous phase and samples were not exposed to Milli-Q or air until the observations on the protein nanoarray were finished. Figure 2 shows height and cross-section-
al TM-AFM images of 500-nm patterns of the PEGDA575 hydrogel at each stage, that is, the hydrated state (Figure 2a,e), the mPEG-S/bPEG-S mixed SAM-modified state (Figure 2b,f), the SA-immobilized state (Figure 2c,g) and the anti-bHSA-immobilized state (Figure 2d,h). As shown in Figure 2a and e, the NW depth of hydrated PEGDA575 increased by about 20 nm compared to dry PEGDA575 (Figure 1c and f). However, neither surface roughness nor the largest lateral swelling possible were observed. In addition, after a period of up to one week in a 10 mM PBS solution, the nanopattern of the PEGDA575 hydrogel was not delaminated from the gold substrate, even though it was swollen (34% in height) by hydration. This result suggests that the acrylate groups of BAC on gold substrates are strongly bound to PEGDA groups by UV irradiation and BAC is a suitable material for the adhesion of PEG-acrylate hydrogels on gold substrates.

The stepwise self-assembled protein nanoarrays with a nanopattern of hydrated PEGDA575 hydrogel were initiated by modifying the mPEG-S/bPEG-S mixed SAM, the self-assembly was then followed through observation in a 10 mM PBS solution. Figure 2b and f shows AFM images of a PEGDA pattern treated with the mPEG-S/bPEG-S mixed SAM. The NW depth of PEGDA575 hydrogel decreased by 3.8 nm compared to the hydrated state and mPEG-S/bPEG-S adsorbate was not found on the PEGDA575 surface. These results indicate that the gold substrate patterned with PEGDA575 hydrogel was selectively modified by the mPEG-S/bPEG-S mixed SAM.

The SA solution was added continuously to the PEGDA patterned substrate that had been modified with the mPEG-S/bPEG-S mixed SAM. In the PEGDA575 NW, both contrast and surface roughness increased with the addition of the SA solution (Figures 2c and g). In the SA-immobilized state, NW depth decreased by 3.8 nm compared to the mixed SAM-modified state, indicating that the SA was confined to the NW of PEGDA575 hydrogel. As shown in Figure 2d and h, after immobilization of anti-bHSA, both contrast and unevenness in the PEGDA575 NW became clearer than in the SA immobilized state. The difference in NW depth between the anti-bHSA- and SA-immobilized states was 8.64 nm. Protein adsorbates were not observed on the PEGDA575 surface during the array process. To verify these results, the size of thiol-functionalized PEG, SA, and anti-bHSA in 10 mM PBS solution were measured as 3.9, 3.5, and 9.1 nm, respectively, using the noninvasive backscattering method. This is in good agreement with the differences in NW depths at each stage. The results suggest that the mPEG-S/bPEG-S mixed SAM and proteins were selectively and gradually self-assembled into the PEGDA575 hydrogel NWs on gold substrates. It is noteworthy that the differences in NW depths at each stage were the maximum differences measured experimentally at three different locations on the same substrate.

Figure 1. a–c) Height and d–f) cross-sectional TM-AFM images of 500-nm patterns of PEGDA575 hydrogel on gold substrates in air. a,d) Mold-detached state after being prebaked at 80 °C for 10 min. b,e) Mold-detached state (Scheme 1a, stage III), and c,f) O2 RIE finished state (Scheme 1a, stage IV) after being prebaked at 100 °C for 10 min.
To confirm the above results, the same process of protein nanoarray construction was investigated with dry PEGDA575 hydrogel in air. After preparing substrates with the same NW depth (57.3 nm) as the PEGDA575 hydrogel, stepwise molecular self-assembly was initiated at the same time in 10 mM PBS solutions. At each stage, the substrates were removed from the PBS solution after 30 min and the samples were observed by AFM in air after gentle drying with an air gun for 5 s. The thickness of the mPEG-S/bPEG-S mixed SAM was estimated using the polymer lift-off method.\(^{[31]}\)

The micropattern of the mPEG-S/bPEG-S mixed SAM was fabricated by combining NIL and PMMA lift-off (Figure 3a and d), and the thiol-functionalized PEGs were successfully grafted onto the gold substrates. The thickness of the mPEG-S/bPEG-S mixed SAM in air was 3.2 nm (Figure 3d), smaller than the values measured by AFM (3.8 nm) and noninvasive backscattering (3.9 nm) in PBS solution, which suggests that the dehydrated PEG mixed SAM is thinner than hydrated PEG mixed SAM under the same grafting time. Figure 3b and e shows height and cross-sectional AFM images of the SA-immobilized state on gold substrates patterned with PEGDA575 in air. The contrast of the SA-immobilized state in the NW was clearer than the same state observed in the PBS solution (Figure 2c and g). The height from the PEGDA575 surface to SA in the NW was 50.6 nm. Therefore, taking into account the 3.2-nm thickness of the PEG mixed SAM and the SA size of 3.5 nm, the NW heights of both the dry PEGDA575 and the dehydrated PEGDA575 from the PBS solution were the same at 57.3 nm. The SA size of 3.5 nm in air is in accordance with the size measured by AFM (3.8 nm) and noninvasive backscattering (3.5 nm) in PBS solution. After affinity binding of anti-bHSA to SA (Figure 3c and f), both the contrast and roughness in the NW were clearer than the SA-immobilized state. The size of anti-bHSA was calculated to be 6.4 nm from the cross-sectional image, which is smaller than the values estimated

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Figure 2. a–d) Height and e–h) cross-sectional TM-AFM images of the 500-nm patterns of PEGDA575 hydrogel on gold substrates in 10 mM PBS solution. a,e) Hydrated state, b,f) PEG mixed SAM-modified state, c,g) SA-immobilized state, and d,h) anti-bHSA-immobilized state. Scale bars: 1 μm.
by AFM in PBS solution (8.64 nm) and by noninvasive backscattering (9.1 nm). The reason for the size difference in anti-bHSA between the PBS solution and air may be related to the orientation because the immunoglobulin is not a globular protein and its length differs with unit-cell angles. It is believed that the Y-shaped immunoglobulin (anti-bHSA) was driven into the SA surface by dehydration stress.

The size of the PEGDA575 NW was further reduced using the same procedure. The well-defined NW patterns of the PEGDA575 hydrogel were successfully replicated down to the feature size of 100 nm by UV-NIL. Protein nanoarrays were formed by the same stepwise self-assembly procedure. Figure 4 shows AFM height images of NWs of various sizes on gold substrates for the anti-bHSA-immobilized state in air. The periodic protein nanopatterns were only constructed in the PEGDA575 NWs. The contrast of arrayed proteins is clear for the 300-nm pattern and selective immobilization of anti-bHSA in the PEGDA575 NWs could also be observed for the sub-200-nm patterns, although the contrast was faint at the smaller NW size. Probe protein (anti-bHSA) nanoarrays with 100-nm feature size were accomplished, as shown in Figure 4d. These results suggest the feasibility of constructing a protein nanoarray with a feature size of sub-100 nm using UV-NIL. Single protein arrays could be constructed by decreasing the size of the PEGDA NWs using UV-NIL and by controlling the molar ratio of the PEG-based biospecific ligand via simple mixing. The success or failure of protein nanoarrays on patterned substrates is determined by the selection of an appropriate inert material combined with further application and the creation of addressability.

In conclusion, we demonstrated a novel method for constructing protein nanoarrays in a nanopatterned PEG hydrogel on a gold substrate. UV-NIL was used to fabricate micro- and nanostructures of PEGDA, which acted as an inert barrier against nonspecific adsorption of thiol-functionalized PEG mixtures (mPEG-S and bPEG-S) as well as proteins (SA and anti-bHSA). The protein nanoarray in the PEGDA575 NW was constructed with a minimum feature size of 100 nm using the stepwise molecular self-assembly. The sizes of thiol-functionalized PEG, SA, and anti-bHSA at each step were confirmed in both a PBS solution and air by AFM measurement and by noninvasive backscattering in a PBS solution. Direct patterning of inert materials using UV-NIL is simple and efficient for constructing protein nanoarrays using stepwise self-assembly, and could be a valuable tool for high-throughput applications such as nanodevices or nanochips.
Gold substrates were prepared by sputtering high-purity Au (99.999%) onto cleaned SiO2 wafers with a titanium adhesion layer (100 nm Au and 5 nm Ti). The substrates were cleaned with UV-ozone treatment for 30 min using an ozone cleaner (NL-150; NM-401; Meisyo Kiko, Japan) equipped with a UV lamp (Toscure251; Toshiba, Tokyo, Japan). The mold was then re-attached with the aid of a fluid cell and a V-shaped silicon nitride cantilever. Data were processed using SPIP V3.3.7.0 software (Image Metrology, Lyngby, Denmark). The sizes of thiol-functionalized molecules (Scheme 1b, stage II) were confirmed by the noninvasive backscattering method (Zetasizer Nano ZS; Malvern Instruments Ltd., Malvern, UK).

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Figure 4. Height TM-AFM images of a) 300 nm, b) 200 nm, c) 120 nm, and d) 100 nm patterned PEGDA575 hydrogel on gold substrates at the anti-bHSA-immobilized state in air. Scale bars: 500 nm.


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